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# Evaluation of doxycycline sensitivity *in vitro* against *Babesia gibsoni* by Real Time-PCR

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## Abstract

Doxycycline sensitivity against *in vitro* cultured *Babesia gibsoni* was evaluated by real-time PCR and parasitemia. The culture of *B. gibsoni* was successfully continued and mean parasitemia was 4.63% when *in vitro* drug sensitivity test was started. The drug sensitivity test by the real-time PCR calculated the gene copy number from cultured sample. Even if the complete growth inhibition was at certain concentration, the genomic DNA might remain in existence. The results revealed that the doses of doxycycline 8µM, 16 µM, 32 µM and 64 µM of both real-time PCR and parasitemia inhibited the growth of *B. gibsoni* in a dose-dependent manner at 96 h and 144 h. We determined the IC50 of doxycycline 17.9 µM at 96 h and 13.8 µM at 144 h after treatment using real-time PCR. Morphological observation revealed that the number of parasites decreased per erythrocyte and line shaped parasites increased in erythrocytes with the increased concentration of doxycycline. Doxycycline effectively inhibits the growth of *B. gibsoni in vitro*. Therefore, doxycycline can be used to treat *B. gibsoni* infection. Further studies are important to know the doxycycline resistance in *B. gibsoni.*

**Keywords:** Doxycycline, *Babesia gibsoni,* RT-PCR, Growth inhibition

## Introduction

*B. gibsoni* is a tick-borne intra-erythrocytic protozoan parasite. It is one of the most common causal agents of severe canine haemolytic anemia. Dogs typically present with the acute form of babesiosis characterized by fever, pallor mucous membrane, lymphadenopathy, splenomegaly, and eventual death (Boozer and Macintire 2003). Chronic infection is common. Definitive diagnosis is based on the clinical signs, history and the identification of *Babesia* spp. within the erythrocytes, positive serologic results and amplification of nucleic acid extracted from blood or tissues (Birkenheuer *et al.,* 2004). Several drugs have been used for the clinical management of canine babesiosis including diminazene aceturate, imidocarb diproprionate (Fowler *et al.,* 1972), pentamidine isethionate phenamidine isethionate (Farwell *et al.,* 1982). Although, these drugs show clinical effectiveness slowly (Suzuki *et al.,* 2007; Lin and Huang, 2010) but these drugs have some adverse effects such as pain at the injection site and nervous symptom due to cerebral haemorrhage (Wulansari *et al.,* 2003). However, no report has discussed the effectiveness of other clinically usable drugs that are available. Some new therapies with antibiotics like clindamycin, metronidazole, doxycycline enrofloxacin have been reported to reduce parasitemia in experimentally infected and in clinical patients (Suzuki *et al.,* 2007; Lin and Huang 2010). Tetracycline antibiotics have been recognized to exhibit activity against protozoa such as *Ehrlichia canis* and *P. falciparum*. In *Babesia* parasites, the activity against *B. divergens* (Losson and Patz, 1989) and *Babesia canis* has been recognized only *in vivo* (Vercammen *et al.* 1966). Most dogs recovered from the acute stage of the disease are at risk of recurrence and may become a reservoir for tick-borne infections (Conrad *et al.,* 1991). Although these therapeutic modalities are beneficial and seem to cure partially, the treatment failed for some of the dogs. Few studies have evaluated the effect of extracts of traditional Indonesian medicinal plants against *B. gibsoni* by using *in vitro* culture and the results of these studies are promising from the view point of future anti-babesial drug development (Groves *et al*., 1972; Murnigsih *et al.,* 2005; Suzuki *et al.,* 2007). To evaluate the effect of potential drugs against this pathogen, an *in vitro* susceptibility test would be important by Real Time-PCR (RT-PCR) but limited information is available. Because *in vitro* culturing of this parasite is difficult and it is successfully conducted only in a limited number of research institutes (Sunaga *et al*., 2002; Murase *et al*., 1991). *B. gibsoni* infected dogs either clinical patient or experimental are required for this method. Our laboratory established a continuous culture of *B. gibsoni* isolated from Tosa dogs (Matsuu *et al*., 2004a). There is no precise report emphasizing the efficacy of doxycycline sensitivity alone *in vitro* against *B. gibsoni* by RT-PCR. In this study, the growth inhibitory effect of different concentrations of doxycycline against *B. gibsoni* tested in a continued culture and evaluated by RT-PCR at different time points. Also the IC50 of doxycycline was determined.

## Materials and Methods

### Test compound

Doxycycline hydrochloride was purchased from Sigma-Aldrich (Tokyo, Japan). Different doses of doxycycline (DXC) 2µM, 4µM, 8µM, 16µM, 32µM and 64µM were used to evaluate the growth inhibitory effects.

### Packed cell volume and thrombocyte count

Measurements of packed cell volume (PCV) and thrombocyte counts were performed using an automated blood cell counter (Celltaq MEK6400, Nihon Kohden Co, Tokyo, Japan).

### Culture of *B. gibsoni*

*B. gibsoni* parasites were isolated from a naturally infected Tosa dog in Aomori Prefecture, Japan (Matsuu *et al*., 2004a). The 18S rDNA sequences of this parasite exhibit 100% homology with the genotype of *B. gibsoni* isolated from dogs in Asia, Oklahoma, North Carolina and Okinawa (Matsuu *et al*., 2004b). The parasite was maintained in the laboratory by passage in beagle dogs.

### Preparation of erythrocytes from the infected blood sample for culture

The blood sample was washed 3 times with phosphate-buffered saline (PBS) by centrifugation at 4 × *g* for 10 min at 4 °C. The culture medium was RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 25 mM pyruvic acid, 2 mM L-glutamine, 100 units/mL penicillin G, 100 µg/mL streptomycin (Invitrogen) and 10% fetal bovine serum (FBS; Funakoshi, Tokyo, Japan). Canine erythrocytes sufficient to yield a packed cell volume (PCV) of 10% were obtained from a healthy Beagle. Packed infected RBCs (200 µl) were dispensed into 1800 µl of culture medium in each well of a 12-well plate. This plate was incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$ and 5% $O_2$ for 7 days. The supernatant medium (800 µl) was replaced daily by a fresh medium. Sub-culturing was performed 7 days after primary culturing was initiated. Normal fresh erythrocytes were sampled from normal beagles by using sodium citrate as the anticoagulant and immediately washed 3 times with PBS. We transferred 200 µl of the cultured and infected erythrocyte suspension to a new well containing 1620 µl of fresh medium and 180 µl of a normal dog erythrocyte suspension. Subsequent continuous culturing was performed every 5–7 days. The percent parasitemia at subculture was calculated by enumeration of 2000 total infected and uninfected RBC on Giemsa-stained smears under microscope. The ensuing parasitemia was monitored daily as above.

### Growth inhibitory test of doxycycline

For the *in vitro* sensitivity test, *B. gibsoni* cultures that had reached parasitemia approximately 5% (parasitemia before adding drug = Pre ) after the last subculture for 2-3 days were collected in a collection tube. Two hundred mL of these parasitized erythrocyte suspension was distributed per well in 96-well plates in three replicates of each drug concentration. Doxycycline hydrochloride was dissolved in ultra pure water and aliquot was prepared and then added to the culture medium to give the final concentrations of 2µM, 4 µM, 8 µM, 16 µM, 32 µM and 64 µM. This range of drug concentrations were based on the results of preliminary assays conducted at the laboratory. From the aliquots drug was added into each well of the serially diluted doxycycline in culture medium and one well without drug (ND) as control for normal growth of parasite. The plates were incubated in a 5% CO2 incubator at $37^0$C. Every 24 h the medium (80 µL) in each well was aspirated and a fresh solution containing the appropriate concentration of the test drug was added. After 48, 72, 96 and 144 h of incubation from each well 1 µL sample was taken for parsitemia and 1 µL for RT-PCR.

### Real-Time PCR

DNA was extracted using DNA extraction kit (QIAamp DNA Mini Kit; Qiagen, USA) and Real-Time PCR was performed using a standard protocol recommended by the manufacturer (iQ SYBR Green Supermix, Bio-Rad, Japan). One µL of template DNA was added to 19 µL of reaction mixture containing 0.5 µL of each primer, 8 µL ultra pure water, and 10 µL iQ SYBR Green Supermix. The reaction was performed under the following conditions (Mini Opticon, Bio-Rad) $95^0$C for 30s. $95^0$C for 3min, $55^0$C for 5s. A thermal melt profile was built following PCR to identify amplicons as specified, and analysis was performed with analysis software (CFX-Manager, Bio-Rad). The $IC_{50}$ of doxycycline was defined as the concentration required for the 50% reduction in the mean number of parasitized erythrocytes from that of control culture after 96 and 144 h of incubation. Each test was performed in triplicates. IC50 values were expressed as the mean concentration ±SD.

### Parasitemia

A thin smear was prepared, fixed with methanol and stained with Giemsa solution to count the number of parasitized erythrocytes. Parasitemia was monitored by counting the number of parasitized cells among 2000 erythrocytes and expressing this as a percentage.

The sensitivity of doxycycline was evaluated by measuring the rate of growth inhibition. This was calculated by counting the number of parasitized erythrocytes per 2000 erythrocytes in each of the wells containing the drug and that in the control well without drug (ND).

## Results and Discussion

*B. gibsoni* culture was successfully continued. Parasitemia reduced upto 0.5% in few phases but recovered and cultured parasite grew steadily and gained considerable size as a result of continuous culturing (Fig. 1).

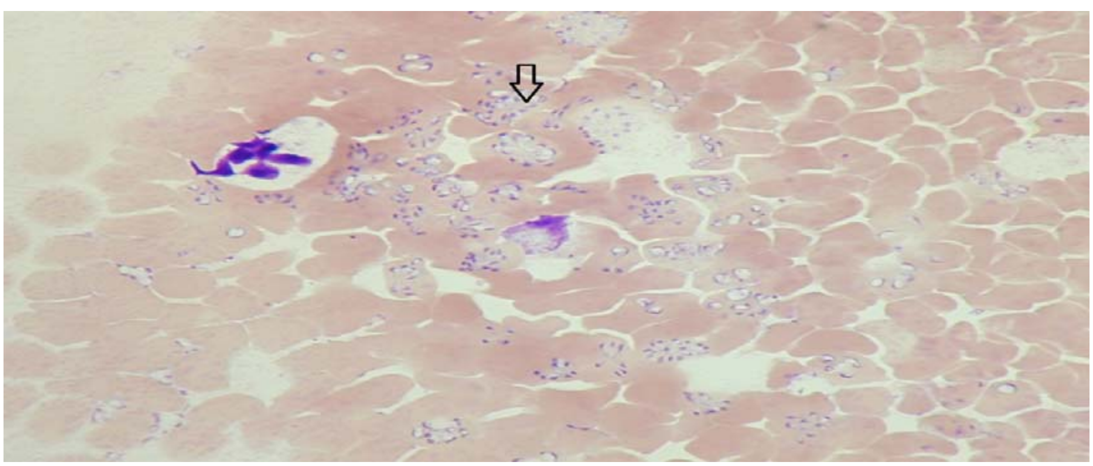

Fig 1. The arrow showing the growth of *Babesia gibsoni* in the sub-culture under microscope (10x)

The rate of growth inhibition of *B. gibsoni* culture has been shown in a tabular form ( Table 1).

**Table 1. The percentages (%) of parasitemia are shown as *in vitro* doxycycline sensitivity against *Babesia gibsoni***

| Drug groups | 48 h (Mean±SD) | 72 h (Mean±SD) | 96 h (Mean±SD) | 144 h (Mean±SD) |
|---|---|---|---|---|
| Pre | 4.633±1.528 | | | |
| ND | 4.80±0.778 | 5.066±1.189 | 6.433±1.247 | 7.766±0.286 |
| 2 µM | 4.30±1.042 | 4.333±0.784 | 6.533±1.265 | 6.821±0.153 |
| 4µM | 4.20±0.668 | 4.533±1.225 | 6.30±1.555 | 7.066±1.020 |
| 8µM | 3.833±0.974 | 3.0±1.042 | 3.233±0.188 | 2.466±0.377 |
| 16µM | 2.466±0.880 | 1.70±0.883 | 2.433±0.659 | 1.90±0.374 |
| 32µM | 1.266±0.094 | 1.033±0.249 | 0.666±0.094 | 0.50±0.163 |
| 64µM | 0.650±0.150 | 0.50±0.141 | 0.433±0.124 | 0.266±0.047 |

The mean parasitemia was 4.6±1.5% when in vitro drug sensitivity test started and mean parasitemia have been counted at 48 h, 72h, 96 h and 144 h after incubation. The sensitivity was evaluated by growth inhibition, determined from the RT-PCR and parasitemia at 96 h and 144 h in different concentrations of doxycycline treated groups Figs. 2 and 3).

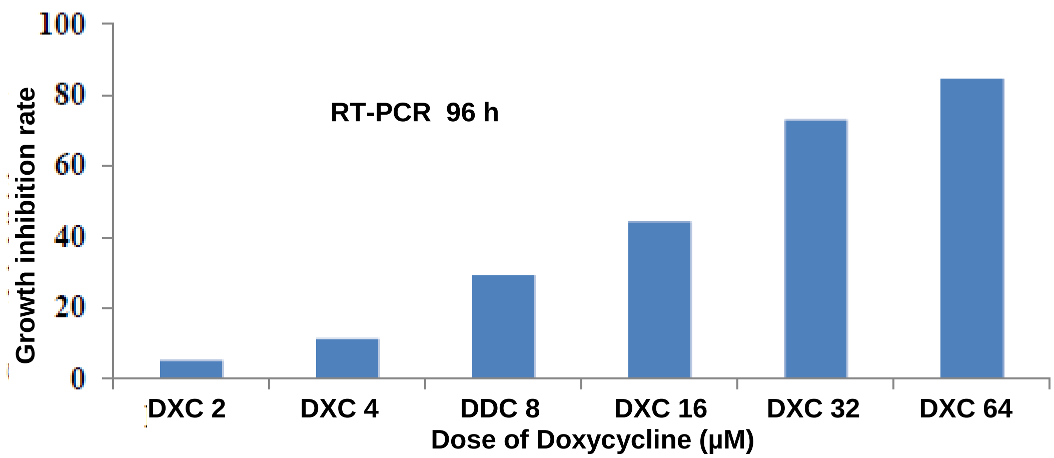


Fig. 2. Growth inhibitory effects of different doses of doxycycline evaluated by RT-PCR at 96 h

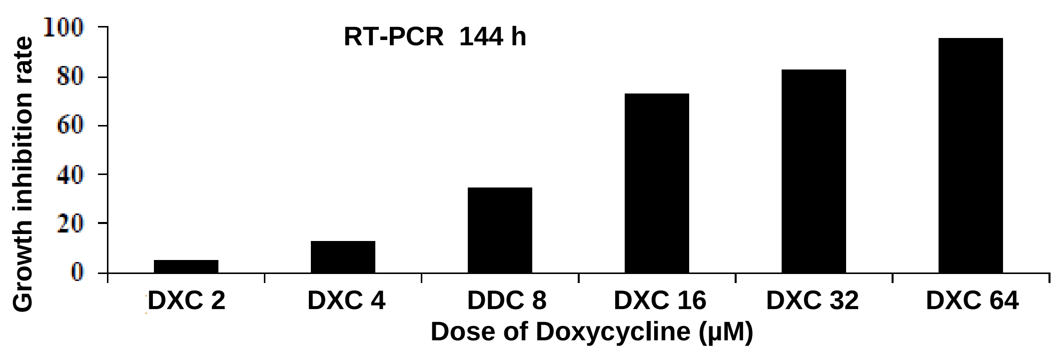


Fig 3. Growth inhibitory effects of different doses of doxycycline evaluated by RT-PCR at 144 h.

The IC50 of doxycycline was found 17.9 µM and 13.8 µM at 96 h and 144 h, respectively. The RT-PCR results revealed that the doses of doxycycline 8µM, 16 µM, 32µM and 64 µM inhibited the growth of *B. gibsoni* in a dose-dependent manner at 96 h (Fig. 2) and at 144 h (Fig. 3). Morphological observation revealed that the number of parasites decreased per erythrocyte and line shaped parasites increased in erythrocytes with the increased concentrations of doxycycline. It was recognized that doxycycline effectively inhibit the growth of *B. gibsoni in vitro*.

Previous studies demonstrated the growth inhibitory effect of different antibiotics including macrolide and lyncomycin against protozoa. These antibiotics probably exhibit inhibitory effects against the apicomplex protozoa by targeting the apicoplast (Vercammen *et al*., 1996). For a long time, tetracycline antibiotics have been recognized to exhibit activity against protozoa such as *Ehrlichia canis* and *P. falciparum*. In *Babesia* parasites, the activity against *B. divergens* (Losson and Patz, 1989) and *Babesia canis* has been recognized only *in vivo* (Vercammen *et al*., 1996). The efficacy of treatment of babesiosis caused by *B. gibsoni* with doxycycline has been evaluated *in vivo* but the ability of this drug alone was controversial. This study will help to understand the growth inhibitory effect of doxycycline by RT-PCR in a better way. Doxycycline provided satisfactory prophylaxis against experimental infection with a highly pathogenic strain of *Babesia canis* but asymptomatic infection could not be ruled out. Imidocarb provides a rather short protection period (Vecammen *et al*., 1996) and has some practical inconvenience regarding availability and administration mode. In addition, terracyclines are also useful against canine ehrliciosis. The IC50 value for doxycycline was slightly higher for *P. falciparum* than for *B. gibsoni*, which was previously reported to be 10.2–17.0 and 7.7–14.9 µM, respectively (Pradines *et al.,* 2001).

It is concluded that treatment of canine babesiosis by oral administration of doxycycline can be of practical importance. Further studies are important to evaluate the doxycycline resistance gene in *B. gibsoni.*

## References


Boozer, AL and Macintire, DK 2003. Canine bebesiosis. *Veterinary Clinics of North American Small Animmal. Practitioner*. 33: 885-904.

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G. and Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. *Veterinary Parasitology*. 124: 151–160.

Conrad, P.A., Thomford, J., Yamane, I., Whiting, J., Bosma, L., Uno, T., Holshuh, H.J. and Shelly, S., 1991. Hemolytic anemia caused by *Babesia gibsoni* infection in dogs. *Journal of American Veterinary Medical Association*. 199: 601–605.

Farwell G. E, LeGrand E. K, and Cobb, C.C. 1982. Clinical observation on *Babesia gibsoni* and *Babesia canis* infections in dogs. *Journal of American Veterinary Medical Association*. 180, 507-511.

Fowler, JL; Ruff MD, Fernau, RC and Furusho, Y.1972. *Babesia gibsoni*: Chemotherapy in dogs. *American Journal of Veterinary Research*. 33; 1109-1114.

Groves, MG and Dennis, GL. 1972. *Babesia gibsoni*: filed and laboratory studies of canine infections. *Experimental Parasitology*. 31, 153-159.

Lin Mig-Yu and Huang Hui-Pi. 2010. Use of doxycycline-enrofloxacin-metronidazole combination with/without diminazene diaceturate to treat naturally occurring canine babesiosis caused by *Babesia gibsoni. Acta Veterinaria Scandenivica*.52:27-30.

Losson, B and Patz R, 1989. Babesia divergens: activity of long-acting oxytetracycline in a gerbil, Meriones unguiculatus. *Annual Record of Veterinary*. 20:501-507.

Matsuu A, Kawabe A, Koshida Y, Ikadai H, Okano S. and Higuchi S. 2004a. Incidence of canine Babesia gibsoni infection and subclinical infection among Tosa dogs in Aomori Prefecture, Japan. *Journal of Veterinary Medical Science*: 66(8) 893-97.

Matsuu A, Koshida Y, Kawahara M, Inoue K, Ikadai H, Hikasa Y, Okano S. and Higuchi S. 2004b. Efficacy of atovaquone against *Babesia gibsoni in vivo* and *in vitro*. *Veterinary Parasitology*. 124:9-18.

Murase T, Hashimoto T, Ueda T, Maede Y. 1991. Multiplication of Babesia gibsoni in in vitro culture and its relation to hemolysis of infected erythrocytes. *Journal of Veterinary Medical Science.* 53(4):759-60.

Murnigsih T, Subeki, Matsuura H, Takahashi K, Yamasaki M, Yamato O, Maede Y, Katakura K, Suzuki M, Kobayashi S, Chairul and Yoshihara T. 2005. Evaluation of the inhibitory activities of the extracts of Indonesian traditional medicinal plants against Plasmodium falciparum and Babesia gibsoni. *Journal of Veterinary Medical Science*. 67(8):829-31

Pradines B, Fusai T, Rogier C, Keundjian A, Sinou V, Merckx A, Mosnier J, Daries W, Torrentino and M, Parzy D. 2001. Prevention and treatment of malaria: in vitro evaluation of new compounds. *Annals of Pharmacology France*.59(5):319-23.

Rashid HB, Chaudhry M, Rashid H, Pervez K, Khan MA and Mahmood AK. 2008. Comparative efficacy of diminazene diaceturate and diminazene aceturate for the treatment of babesiosis in horses. *Tropical Animal Health and Prododuction*., 40: 463-467.

Sunaga, K. Namikawa and Y. Kanno. 2002. Continuous in vitro culture of erythrocytic stages of *Babesia gibsoni* and virulence of the cultivated parasite. *Journal of Veterinary Medical Science*. 64: 571–575.

Suzuki K, Wakabayashi H, Takahashi M, Fukushima M, Yabuki A and Endo Y. 2007. A possible treatment strategy and clinical factors to estimate the treatment response in *Babesia gibsoni* infection. *Journal of Veterinary Medical Science.* 69: 563-568.

Vercammen F, De Deken R and Maes L. 1996. Prophylactic treatment of experimental canine babesiosis (*Babesia canis*) with doxycycline. *Veterinary Parasitology*, 66:251-255.

Wulansari R, Wijaya A, Ano H, Horii Y, Nasu T, Yamane S and Makimura S. 2003. Clindamycin in the treatment of *Babesia gibsoni* infections in dogs. *Journal of American Animal Hospital Association*. 39:558-562.